

Applicants: Ridwan Shabsigh
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REMARKS

Claims 9 and 10 are pending in the subject application. By this Amendment, Applicant has added claims 12-21. Support for new claims 12-21 may be found, *inter alia*, in the specification as follow: Claim 12: Page 13, lines 17-24; and page 11, lines 20-26; Claim 13: Page 13, lines 17-24; and page 12, lines 9-35; Claim 14: Page 13, lines 17-24; and page 12, lines 1-7; Claim 15: Page 13, lines 17-24; page 12, lines 1-7; and page 14, lines 3-21; Claim 16: Page 13, lines 17-24; page 12, lines 1-7; and page 11, lines 20-26; Claim 17: Page 13, lines 17-24; and page 12, lines 1-7; Claim 18: Page 13, lines 17-24; and page 12, lines 1-7; Claim 19: Page 13, lines 17-24; page 12, lines 1-7; and page 14, lines 3-21; Claim 20: Page 13, lines 17-24; page 12, lines 1-7; and page 11, lines 20-26; Claim 21: Page 13, lines 17-24; page 12, lines 1-7; and page 12, lines 9-35.

Accordingly, claims 9-10 and 12-21 will be pending and under examination upon entry of this Amendment.

Rejection Under 35 U.S.C. § 112, First Paragraph:

The Examiner maintained the rejection of claims 9 and 10 as allegedly failing to comply with the enablement requirement. Specifically, the Examiner alleged that Applicant did not describe in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

In response, Applicant respectfully traverses the Examiner's ground of rejection and maintains that claims 9 and 10 satisfy

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the enablement requirement of 35 U.S.C. § 112, first paragraph.

The test for enablement is whether one skilled in the art could, at the time of the invention, make and use the claimed invention based on the disclosure and information known in the art without undue experimentation.

Applicant notes the Examiner's assertion that (i) there is a lack of predictability that VEGF would increase vascularization and therefore blood supply; and (ii) based on the prior art, a person having skill in the art would conclude that it was not reasonably predictable that any particular gene therapy would work to increase or maintain the blood supply in the subject's penis.

Applicant disagrees with the Examiner's position. First, Applicant has explained that at the time the application was filed, it was known that penile vascular insufficiency is a mechanism of erectile dysfunction. Such teaching can be found in the specification at, *inter alia*, page 1, line 27 through page 2, line 2. Further, Applicant pointed out that there are VEGF-encoding genes that play a significant role in angiogenesis. Such teaching can be found in the specification at, *inter alia*, page 3, line 6 through page 4, line 1. In addition, Applicant explained that an increase of VEGF in tissue levels results in an increase in tissue vascularity. Such teaching can be found in the specification at, *inter alia*, page 2, lines 15-31. The specification also discloses in the specification at, *inter alia*, page 27, line 15 through page 28, line 22, Applicant's discovery that VEGF is expressed

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in the mammalian penis. Finally, the Applicant discloses in the specification at, *inter alia*, page 12, line 37 through page 13, line 15, specific routes of VEGF administration. Accordingly, Applicant maintains that the subject specification teaches how to practice the claimed method without undue experimentation.

The Examiner referred to the Burchardt reference of record for the following passage: "Although statistical trends were measured in the VEGF protein-treated group, no statistically significant difference in smooth muscle or endothelial cell content was found between control and VEGF-treated rats." Further, the Examiner noted that in Burchardt the amount of smooth muscle cell and endothelial cell area is not different between protein and DNA transfer protocols, and that Burchardt teaches that VEGF likely requires other factors to act in concert and over time to provide angiogenic benefits.

Applicant respectfully submits that the Examiner's reliance on Burchardt does not support the enablement rejection. The lack of statistical significance in Burchardt between the smooth muscle and endothelial tissue in control and VEGF-treated rats does not contradict Applicant's teaching. VEGF administration has in fact been shown to increase or maintain blood supply and to ameliorate erectile dysfunction in mammals as demonstrated in Ming-Chan Lee, et al., annexed hereto as **Exhibit A**. In short, Ming-Chan Lee, et al., teach that "a single bolus of VEGF injection [is] effective for improving blood flow into the target organ."

Further, Burchardt reflects measurements in normal animals,

and the experiment is performed for the purpose of demonstrating the predictability of the gene therapy. The animals used in Burchardt do not exhibit a disease state such as vascular insufficiency. Therefore, normal animals would not be expected to react to a treatment in the same way as animals exhibiting a disease state, such as the animals with arteriogenic erectile dysfunction of Ming-Chan Lee, et al.

Burchardt demonstrates that gene therapy can be achieved without undue experimentation, and that the amount of vascular endothelial growth factor in the penis can be increased by such gene therapy. Further, Ming-Chan Lee, et al. demonstrate that an increased amount of VEGF in the penis increases blood flow and treats erectile dysfunction.

Finally, R.S. Rogers, et al., annexed hereto as **Exhibit B**, evaluate VEGF gene therapy and protein therapy in preventing and reversing the development of erectile dysfunction in rat models of venogenic dysfunction. R.S. Rogers, et al. report treatment of rats with venogenic erectile dysfunction with intracavernosal VEGF or an adeno-associated virus vector mediated VEGF gene therapy (AAV-VEGF). Animals treated with either VEGF or AAV-VEGF at the time of castration maintained erectile function. The animals treated with placebo did not maintain erectile function after castration. R.S. Rogers, et al. clearly confirm the use of VEGF gene therapy to treat vasogenic erectile dysfunction.

Therefore, a significant body of evidence supports enablement of Applicant's claimed invention.

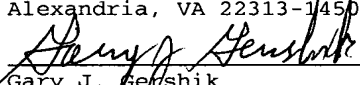

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In view of this body of evidence supporting Applicant's invention, claims 9, 10, 11, and 12 are enabled; new claims 14-17 directed to a method for increasing the amount of VEGF in the penis are enabled; and new claims 18-21 directed to a method for treating erectile dysfunction are enabled. Accordingly, Applicant maintains that the claimed invention is enabled and respectfully requests that the Examiner reconsider and withdraw this ground of rejection.

If a telephone interview would be of assistance in advancing prosecution of the subject application, Applicant's undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the enclosed \$395.00 fee for filing an RCE, is deemed necessary. However, if any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to:	
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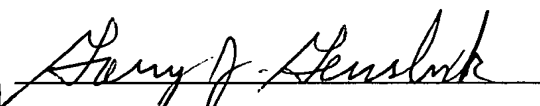

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EXHIBIT A

THE EFFECT OF VASCULAR ENDOTHELIAL GROWTH FACTOR ON A RAT MODEL OF TRAUMATIC ARTERIOGENIC ERECTILE DYSFUNCTION

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ABSTRACT

Purpose: We tested the hypothesis that intracavernous injection of vascular endothelial growth factor (VEGF) can restore erectile function in a rat model of traumatic arteriogenic erectile dysfunction.

Materials and Methods: Exploration of bilateral internal iliac arteries was performed in 50, 3-month-old male rats. A total of 44 rats underwent bilateral ligation of the internal iliac arteries and 6 that underwent exploration only served as the sham operated group. Minutes later intracavernous injection of phosphate buffered saline (PBS) plus bovine serum albumin in 16 rats, 2 μ g. VEGF plus PBS plus BSA in 12 and 4 μ g. VEGF plus PBS plus BSA in 16 was performed. At weeks 1, 2 and 6 about a third of the rats in each group underwent electrostimulation of the cavernous nerves to assess erectile function and were then sacrificed. Penile tissues were collected for histochemical and electron microscopy examinations.

Results: No impairment of erectile function was noted in sham operated rats. Immediately after arterial ligation all rats showed little or no erectile response to neurostimulation. In PBS treated rats modest recovery of erectile function was noted at week 6. Significant recovery of erectile function was noted in VEGF treated rats at weeks 1 and 2 in the 4 μ g. group only and at week 6 in the 2 and 4 μ g. groups. Neuronal nitric oxide synthase staining showed a reduction in neuronal nitric oxide synthase positive nerve fibers in the dorsal or intracavernous nerves at week 1. Moderate recovery of neuronal nitric oxide synthase positive nerve fibers was noted in the 2 and 4 μ g. VEGF treated groups but not in the PBS treated group. Electron microscopy revealed no pathological change in sham operated rats. In dorsal nerves the atrophy of myelinated and nonmyelinated nerve fibers was noted in ligated plus PBS treated rats. Partial recovery was observed in VEGF treated rats. Scattered atrophic smooth muscle cells were seen in PBS and occasionally in VEGF treated rats but not in the sham operated group. The most dramatic findings in VEGF treated rats were hypertrophy and hyperplasia of the endothelial cells, especially those lining the small capillaries.

Conclusions: Ligation of bilateral internal iliac arteries produced a reliable animal model of traumatic arteriogenic erectile dysfunction. Intracavernous injection of VEGF minutes after arterial ligation facilitated the recovery of erectile function.

KEY WORDS: penis; impotence; muscle, smooth; rats, Sprague-Dawley; endothelial growth factors

Penile arterial insufficiency is one of the most common causes of erectile dysfunction. Atherosclerotic or traumatic arterial occlusive disease of the pudendal-cavernous-helicine arterial tree can decrease perfusion pressure and arterial flow to the sinusoidal spaces, thus, decreasing the rigidity of the erect penis. Common risk factors associated with general arterial insufficiency include hypertension, hyperlipidemia, cigarette smoking, diabetes mellitus and pelvic irradiation.¹⁻³ Epidemiological studies have shown a high incidence of erectile dysfunction in patients with coronary arterial disease.⁴ Focal lesion of the common penile or cavernous artery

is most common in young patients who have sustained blunt pelvic or perineal trauma, as in bicycle accidents.³

Angiogenesis is a complex process that includes activation, migration and proliferation of endothelial cells, and formation of new blood vessels.⁵ Vascular endothelial growth factor (VEGF) has been shown to be intimately involved in the whole sequence of events leading to the growth of new blood vessels.^{6,7} Seven human VEGF isoforms of 111, 121, 145, 165, 183, 189 and 206 amino acids have been isolated.⁸⁻¹³ Of the isoforms VEGF165 seems to be most effective and most commonly used. The effect of VEGF165 for augmenting perfusion and stimulating the formation of collateral vessels has been shown in animal models.¹⁴⁻²² In clinical trials successful induction of collateral blood vessels in ischemic heart disease and critical limb ischemia by VEGF has also been reported.^{23,24}

In a preliminary study we successfully developed a rat model of traumatic arteriogenic erectile dysfunction by ligating each internal iliac artery. In these rats arteriogenic erectile dysfunction developed immediately without gross isch-

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|| Financial and/or other relationship with Tap and Pfizer.

emic changes in the external genitalia or pelvic organs. When followed for 6 weeks, slight improvement in erectile function occurred but no rats regained complete erection, as assessed by electrostimulation of the cavernous nerves.²⁵ We tested the hypothesis that by reacting with VEGF receptors on the endothelial and smooth muscle cells direct injection of VEGF into the corpus cavernosum can stimulate angiogenesis and enhance the recovery of erectile function after bilateral ligation of the internal iliac arteries.

MATERIALS AND METHODS

Animal model. The experimental protocols and animal care were approved by our institutional Committee on Animal Research. A total of 50, 3-month-old male Sprague-Dawley rats weighing 350 to 400 gm. were anesthetized intraperitoneally with 35 mg/kg. pentobarbital after being sedated by methoxyflurane inhalation. Midline laparotomy was performed to identify the iliac vessels. Under operating microscopy the iliac veins were carefully dissected to expose the 2 to 4 internal iliac arteries. The 44 rats in the experimental group underwent bilateral ligation of the internal iliac arteries at their origin, while those in the sham operated group underwent exploration only. A separate incision was made in the perineum to identify 1 crus. A 23 gauge scalp vein needle was inserted into the crus and connected to a pressure monitor for intracavernous pressure monitoring during electrostimulation of the cavernous nerve. Additional ligation of arterial branches was performed until there was minimal or no intracavernous pressure rise during electrostimulation. Intracavernous injection of 4 μ g. VEGF in phosphate buffered saline (PBS) with 0.1% bovine serum albumin, 2 μ g. VEGF in PBS with 0.1% bovine serum albumin or PBS solution with 0.1% bovine serum albumin was then administered through the same needle to 3 groups of 16, 12 and 16 rats, respectively. The wound was closed in layers and the animals were closely monitored for up to 6 weeks. At weeks 1, 2 and 6 about a third of the rats in each group underwent electrostimulation of the cavernous nerve to assess erectile function and were then sacrificed. Penile tissues of 3 rats randomly chosen from each of the 3 subgroups at 2 and 6 weeks were obtained for immunohistochemical staining and electron microscopy.

Electrical stimulation. Bipolar platinum wire electrodes were used to stimulate the cavernous nerve. The exposed end of the electrodes was hooked around the nerve to be stimulated with the positive electrode positioned proximal and the negative electrode 2 to 3 mm distal. Stimulus parameters were 1.5 V., frequency 20 pulses per second, pulse width 0.2 milliseconds and duration 50 seconds. Intracavernous pressure was monitored and recorded by inserting a number 23 scalp vein needle into a crus and connected to a pressure monitor.

Immunohistochemical staining. Penile tissue was fixed for 3 hours in a cold, freshly prepared solution of 2% formaldehyde, 0.002% picric acid in 0.1 M. phosphate buffer, pH 8.0. Tissues were cryoprotected for 24 hours in cold 30% sucrose in 0.1 M. phosphate buffer, pH 8.0. They were then embedded in Tissue-Tek OCT. compound (Sakura Finetek, Inc., Torrance, California), frozen in liquid nitrogen and stored at -70°C. After freezing cryostat tissue sections were cut at 10 μ m., adhered to charged slides, air-dried and hydrated for 5 minutes with 0.05 M. PBS, pH 7.4. Sections were treated with hydrogen peroxide/methanol to quench endogenous peroxidase activity. After rinsing with water sections were washed twice in PBS for 5 minutes, followed by 30 minutes of room temperature incubation with 3% horse serum/PBS/0.3% Triton X-100. After draining solution from sections tissues were incubated for 60 minutes at room temperature with mouse monoclonal anti-neuronal nitric oxide synthase (Transduction Laboratories, Lexington, Kentucky), diluted

1:500. After washing for 5 minutes with PBS/Triton X-100 and then for 5 minutes twice with PBS alone sections were immunostained with the avidin-biotin-peroxidase method using an Elite ABC kit (Vector Laboratories, Burlingame, California) with diaminobenzidine as the chromagen, followed by counterstaining with hematoxylin.

Electron microscopy. The penis was dissected, thinly sliced to approximately 1 mm., placed in Karnovsky fixative (1% paraformaldehyde/3% glutaraldehyde/0.1 M. sodium cacodylate buffer, pH 7.4) at room temperature for 30 minutes and then stored at 4°C. The fixed tissue was then rinsed in buffer, post-fixed in 2% aqueous OsO₄ and stained en bloc with uranyl acetate before being dehydrated in ethanol, cleared in propylene oxide and embedded in eponate 12 (Ted Pella Co., Redding, California). Thick sections were cut and stained with toluidine blue and examined under light microscopy to select the area to be thin sectioned. Thin sections were cut by an Ultracut E microtome (Leica, Bannockburn, Illinois), stained with uranyl acetate and Reynold's Lead to enhance contrast and examined by Tecnai 10 electron microscope (Philips, Eindhoven, The Netherlands). Data were evaluated by the Mann-Whitney rank sum test with significance considered at $p < 0.05$.

RESULTS

Erectile function. The table shows the peak sustained intracavernous pressure during electrostimulation of the cavernous nerve. There was no difference in intracavernous pressure in sham operated and normal rats used for other experiments. In all experimental rats after bilateral ligation of the internal iliac arteries intracavernous pressure immediately dropped to around 20 cm. water and produced no or a minimal pressure increase in response to neurostimulation. In the PBS treated group poor erectile response persisted at weeks 1 and 2, and slight recovery of erectile function was noted at week 6.

In VEGF treated rats at weeks 1 and 2 moderate recovery of erectile function was noted in the 4 but not in the 2 μ g. group. At week 6 statistically significant improvement in intracavernous pressure was seen in the 2 and 4 μ g. groups compared with the PBS treated group. Intracavernous pressure in the 4 μ g. group was also significantly higher than in the 2 μ g. group. To identify the new source of blood flow in the 4 μ g. VEGF treated group we noted a decrease in erectile response after clamping 1 external iliac artery and no erectile response after clamping the 2 external iliac arteries, which strongly suggests that the collateral vessels were derived from the external iliac arteries.

Histochemical testing. There was a trend toward decreased neuronal nitric oxide synthase immunoreactivity in the dorsal and intracavernous nerves 2 weeks after arterial ligation in all experimental subgroups (fig. 1). At week 6 moderate

Peak sustained intracavernous pressure during electrostimulation of the cavernous nerves in saline and VEGF treated rats

	PBS + 0.1% Bovine Serum Albumin	PBS, 0.1% Bovine Serum Albumin + 2 μ g. VEGF	PBS, 0.1% Bovine Serum Albumin + 4 μ g. VEGF
Wk. 1:			
Mean pressure \pm SD (cm. water)	20.33 \pm 3.45	23.50 \pm 2.38	71.17 \pm 16.89
No. rats	6	4	6
Wk. 2 mean pressure \pm SD (cm. water)*	27.75 \pm 9.70	43.00 \pm 8.37	86.25 \pm 8.18
Wk. 6:			
Mean pressure \pm SD (cm. water)	46.75 \pm 14.85	69.00 \pm 8.83	96.67 \pm 13.50
No. rats	6	4	6

Sham operated group of 6 rats mean 98 \pm 8.50 cm. water at week 6.

* Four rats per group.

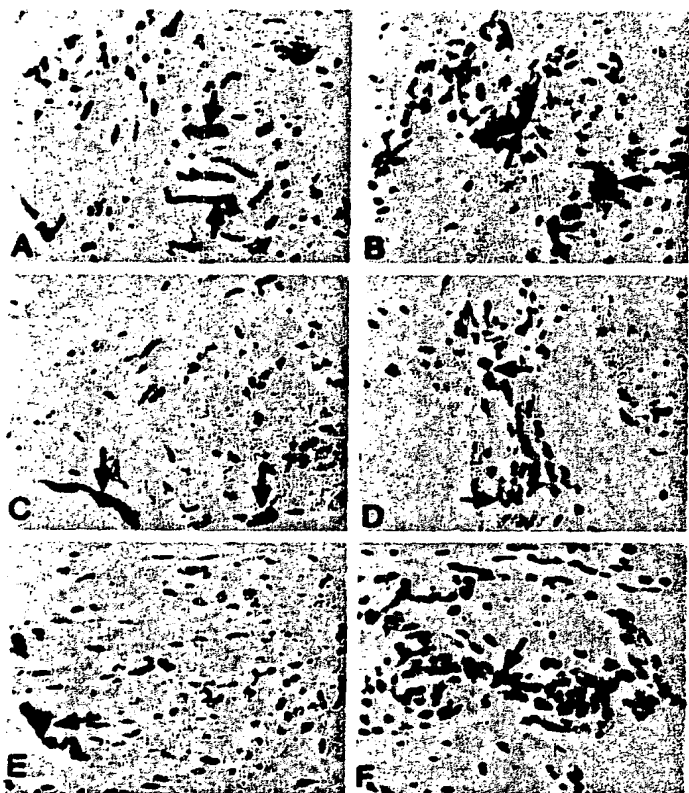


FIG. 1. Neuronal nitric oxide synthase staining of penile tissue in sham operated rat (A and B) and in rats 2 weeks after bilateral iliac artery ligation (C, D, E and F). Abundant neuronal nitric oxide synthase positive nerve fibers (arrows) were noted in dorsal (A) and intracavernous (B) nerves in sham operated rat. There was reduction in neuronal nitric oxide synthase positive nerves in VEGF treated dorsal (C) and intracavernous (D) nerves, and in PBS treated dorsal (E) and intracavernous (F) nerves. Reduced from $\times 400$.

recovery of neuronal nitric oxide synthase positive nerve fibers was noted in the dorsal and intracavernous nerves in the 2 and 4 μg . VEGF treated groups but not in the PBS treated group (fig. 2). However, the differences were not statistically significant on computer assisted image analysis.

Electron microscopy. Dorsal Nerve: No difference was noted in specimens obtained from sham operated rats and normal rats used for other experiments. The dorsal nerve in these rats was filled with myelinated and nonmyelinated nerve bundles. The mean diameter of the individual myelinated nerve axons plus or minus standard deviation was $4.42 \pm 1.36 \mu\text{m}$, excluding the myelin sheath. The mean thickness of the myelin sheath was $0.58 \pm 0.21 \mu\text{m}$. The mean diameter of the nonmyelinated nerve fibers was $0.96 \pm 0.37 \mu\text{m}$. The nuclei of Schwann cells were seen occasionally near nerve fibers (fig. 3, A).

In ligated plus PBS treated rats dramatic changes were noted at week 2 (fig. 3, B). There was an increase in the number of Schwann cells, of which many contained vacuoles within the cytoplasm. There was also a decrease in the number of nonmyelinated and myelinated nerve fibers. Overall the axons were smaller than in controls and many nonmyelinated nerve fibers were smaller and less discrete. At week 6 various degrees of regeneration of myelinated and nonmyelinated nerve fibers were apparent (fig. 3, C). However, the myelinated and nonmyelinated nerve fibers, were still smaller than in the control groups (mean myelinated axon and sheath 3.17 ± 1.01 and $0.46 \pm 0.11 \mu\text{m}$, and mean nonmyelinated axon $0.81 \pm 0.38 \mu\text{m}$, $p = 0.062$, 0.189 and 0.069 , respectively, versus the sham operated group). Many

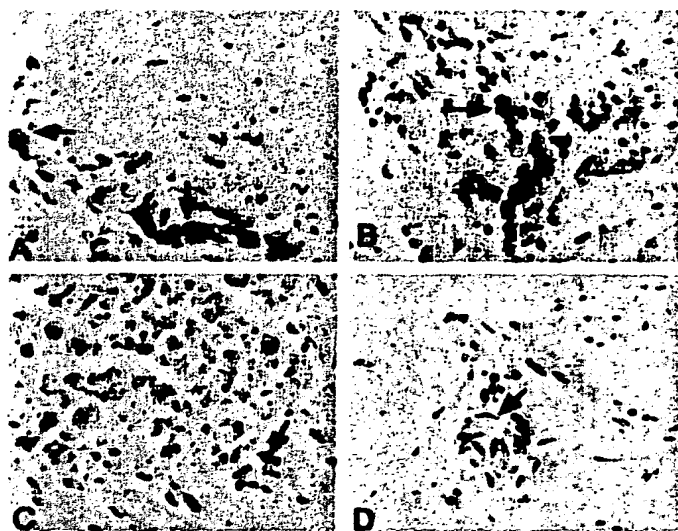


FIG. 2. Neuronal nitric oxide synthase staining of penile tissue 6 weeks after arterial ligation. Moderate recovery of neuronal nitric oxide synthase positive nerve fibers (arrows) in dorsal (A) and intracavernous (B) nerves was noted in 4 μg . VEGF treated group but not in PBS treated group dorsal (C) or intracavernous (D) nerve. Reduced from $\times 400$.

nonmyelinated fibers were less than a third of the size of the larger ones. There was also an increase in the number of nucleated Schwann cells.

At week 6 in ligated rats treated with 4 μg . VEGF the mean diameter of the myelinated axons was larger than in the ligated plus PBS treated rats (6.19 ± 2.38 and $3.17 \pm 1.01 \mu\text{m}$, respectively, $p = 0.008$, fig. 3, D). The mean diameter of the nonmyelinated axons was $0.82 \pm 0.45 \mu\text{m}$, which was similar to that in the ligated plus PBS treated rats ($0.81 \pm 0.38 \mu\text{m}$). However, the nonmyelinated nerve fibers appeared more even in size.

Intracavernous Smooth Muscle: No difference was noted in specimens obtained from sham operated rats and normal rats used for other experiments. The smooth muscle cells, of which most were arranged in clusters, were embedded in fine strands of fibroconnective tissue (fig. 4, A). The cytoplasm of these myocytes contained abundant contractile myofilaments and dense bodies. Occasionally small aggregates of organelles, including mitochondria, rough endoplasmic reticulum and Golgi apparatus, were found adjacent to the nucleus. The cell membrane (sarcolemma) consisted of many alternating dense and light bands with the latter containing numerous pinocytotic vesicles (caveolae). The intercellular spaces were narrow and many cell-to-cell contacts (gap junctions) were visible. Nerve terminal varicosities were occasionally observed near clusters of smooth muscle cells.

At week 2 in ligated plus PBS treated rats the smooth muscle cells appeared to be atrophic and were separated by large amounts of connective tissue. At week 6 the number of normal-appearing smooth muscle cells increased, although many still showed a significant loss of myofilaments (fig. 4, B). In ligated plus 4 μg . VEGF treated rats in the 2 and 6-week groups most smooth muscle cells appeared normal with large amount of myofilaments and narrow intercellular spaces (fig. 4, C).

Endothelium: In sham operated rats the cavernous sinuoids were lined with intact endothelium, of which the cytoplasm contained numerous pinocytotic vesicles (caveolae), mitochondria, rough endoplasmic reticulum and Golgi apparatus (fig. 5, A). The nuclei of the endothelial cells were sparse and appeared flattened. In ligated plus PBS treated

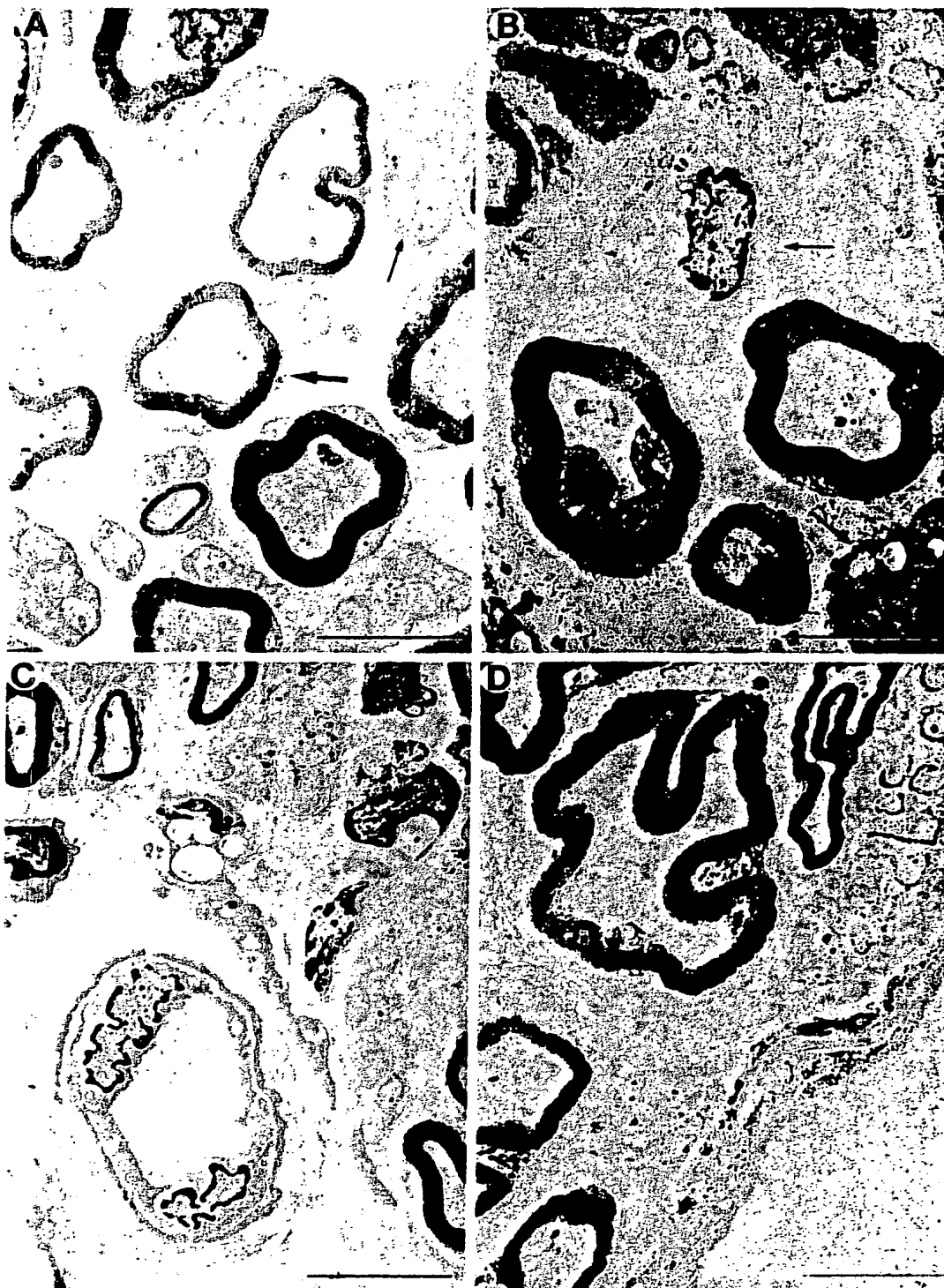


FIG. 3. Representative electron micrographs of cross sections of penile dorsal nerves. In sham operated rats at week 6 dorsal nerve was filled with myelinated (thick arrow) and nonmyelinated (thin arrow) nerve fibers (A). Scale bar indicates 5 μ m. In ligation plus PBS treated rats at week 2 there appeared to be increased number of nucleated Schwann cells (arrows), of which many contained vacuolated and partially degenerated axons within cytoplasm (B). In ligation plus PBS treated rats at week 6 continuing degeneration of myelinated and nonmyelinated nerve fibers was apparent and they were significantly smaller than in sham operated group (C). In ligated plus VEGF treated rats at week 6 large myelinated nerve fibers with thick myelin sheaths were dominant feature (D).

rats in the 2 and 6-week groups the capillary and cavernous sinusoidal endothelium appeared normal, although the number of endothelial cells was increased in some fields (fig. 5, B). In ligated plus 4 μ g. VEGF treated rats in the 2 and 6-week groups striking differences were noted com-

pared with other groups. Many reactive endothelial cells with plump nuclei were visible lining the sinusoids and capillaries. These endothelial cells were larger and more numerous, indicative of endothelial cell hypertrophy and hyperplasia (fig. 5, C).

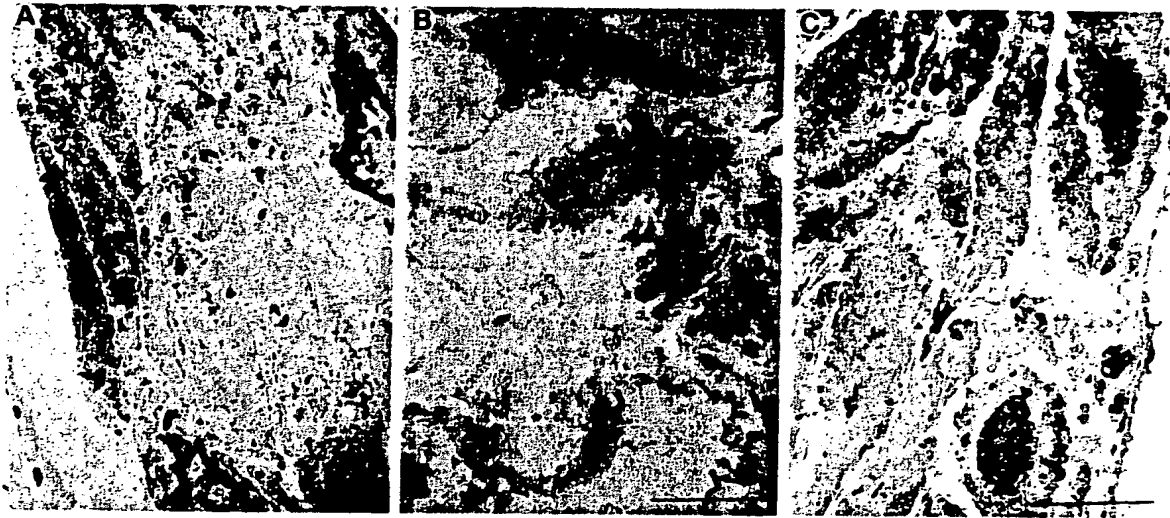


FIG. 4. Representative electron micrographs of cross sections of intracavernous erectile tissues at week 6. In sham operated rats smooth muscle cells were embedded in fine strands of fibroconnective tissue and intercellular spaces were narrow (A). Scale bar indicates 5 μ m. In ligation plus PBS treated rats many atrophic smooth muscle cells separated by large amounts of collagen fibers were noted (B). In ligation plus 4 μ g VEGF treated rats most smooth muscle cells appeared normal with large amount of myofilaments and narrow intercellular spaces (C).



FIG. 5. Representative electron micrographs of cross sections of intracavernous penile tissues at week 6. In sham operated rats capillaries were lined by intact endothelium and occasionally seen nuclei of endothelial cells appeared oval-shaped or elongated (arrow) (A). Scale bar indicates 5 μ m. In ligation plus PBS treated rats capillary endothelium appeared normal, although increased number of endothelial cells was seen in some fields (arrows) (B). In ligation plus VEGF treated rats nuclei of endothelial cells lining many capillaries were large and more numerous, indicating endothelial hypertrophy and hyperplasia (arrows) (C).

DISCUSSION

Although vasculogenic erectile dysfunction in an atherosclerotic rabbit model has been extensively studied,^{26,27} we elected to investigate the feasibility of a smaller animal model of traumatic arteriogenic erectile dysfunction. This rat model offers several advantages. The cavernous nerve can easily be identified and stimulated to assess the erectile response. Rats are less likely to have wound infection and anesthesia related complications than rabbits. The disadvantage of this model is the small size of the internal iliac artery. Complete ligation of the bilateral internal iliac arteries was facilitated by operating microscopy and intracavernous pressure monitoring. Immediately after ligation none of the rats achieved erection in response to electrostimulation, thus, confirming that all branches to the penis had been ligated.

We chose direct intracavernous injection of VEGF for cer-

tain reasons. Local intramuscular injection of VEGF has been reported to induce angiogenesis in the treated limb.^{18,20,21} VEGF165 binds to VEGFR-1 and VEGFR-2 receptors, which are expressed almost exclusively in endothelial cells.⁹ Endothelial cells also express neuropilin-1 and neuropilin-2 receptors, which also bind to VEGF165.⁹ Since billions of endothelial cells exist in the corpus cavernosum, we believe that intracavernous delivery of VEGF can produce the best results. Penile smooth muscle cells in tissue culture also express VEGF receptors and respond positively to VEGF treatment.²⁸ The side effects of systemic VEGF administration, such as hypotension¹⁶ and promiscuous induction of angiogenesis in nontarget tissues, are minimized.²⁹⁻³¹ The results of this study suggest that intracavernous delivery of VEGF may be an efficient method for enhancing the recovery of erectile function after ligation of the internal iliac arteries.

We elected to give a single bolus injection of VEGF because of the difficulty in repeating intracavernous injections into the rat corpus cavernosum, which has a diameter of only about 1 to 2 mm. Nevertheless, a single bolus and 2 boluses of VEGF injection have been shown to be equally effective for augmenting revascularization and improving muscle blood flow and muscle function.^{17, 18, 20, 21, 32} A single bolus of VEGF injection is also capable of significantly augmenting blood flow to ischemic myocardium,¹⁶ accelerating re-endothelialization in balloon injured rat carotid artery,¹⁵ and improving coronary blood flow and preserving regional hemodynamics in chronic ischemic myocardium.³³ Our results confirmed that a single bolus of VEGF injection was effective for improving blood flow into the target organ.

Systemic administration of large doses of VEGF can be quite expensive and may lead to adverse systemic side effects due to unwanted angiogenesis, such as tumor metastasis and pathological retinopathy.³¹ Therefore, most researchers prefer local injection of VEGF into the target organ directly or via its arterial supply. The reported doses of VEGF administered intraarterially to promote neovascularization has been 160 to 3,000 $\mu\text{g}/\text{kg}$.^{17, 19, 32} However, in 1 report a small dose of 2 μg improved coronary flow and preserved regional hemodynamics.³³ Because the corpus cavernosum is replete with billions of endothelial cells, we elected to use small doses of 2 and 4 μg VEGF in the hope that VEGF receptors in the endothelial cells would efficiently react with the injected VEGF.

Even at week 1 the group of rats that received 4 μg VEGF had significantly higher intracavernous pressure in response to neurostimulation. This finding suggests that VEGF may protect penile tissues from ischemic insult caused by ligation of the 2 internal iliac arteries. However, to our knowledge the exact mechanism of this protective effect is unknown. Nevertheless, VEGF is also called vascular permeability factor and microvascular hyperpermeability of plasma proteins has been shown to have an important consequence; namely the laying down of a fibrin-rich extracellular matrix. In turn, this provisional matrix favors and supports the ingrowth of fibroblasts and endothelial cells, which are important for angiogenesis.³⁴ Approximately 50% to 70% of VEGF₁₆₅ binds to heparan sulfate containing proteoglycans in the extracellular matrix and this stored VEGF may be released by suramin, heparin, heparan sulfate, heparinase or plasmin.³⁵ The binding and release of VEGF to and from the extracellular matrix may explain its prolonged angiogenic effect after 1 bolus injection.

In a rabbit model of hindlimb ischemia the functional improvement index and blood pressure ratio were significantly higher in the VEGF treated than in the control group at days 7, 10 and 15 after the delivery of VEGF. At day 30 the blood pressure ratio further improved in the VEGF treated group.²¹ Angiography also revealed that collateral arteries had developed significantly by day 30.²¹ Furthermore, endothelial repair has been shown to be 80% complete 14 days after VEGF treatment.¹⁵ These reports confirm our study, in that the response to neurostimulation improved continuously up to 6 weeks when the last groups of VEGF treated rats were sacrificed.

Our histological findings suggest that functional impairment after bilateral ligation of the internal iliac arteries was a result of the degeneration of neuronal nitric oxide synthase containing nerves. However, our electron microscopy examinations revealed that the ischemic insults also caused significant changes in myelinated and nonmyelinated nerves as well as in smooth muscle cells, of which all are important regulators of penile erection. After VEGF treatment the regeneration of nerves and smooth muscles were evident. Although we were unable to show the regrowth of new blood vessels, hyperplasia and hypertrophy of endothelial cells lining the capillary and sinusoidal endothelium after VEGF

treatment provided indirect evidence of angiogenesis, which may be the source of tissue regeneration.

To our knowledge the exact mechanism by which VEGF improves erectile function is unknown. Nevertheless, we observed clear evidence of restoration of neural and smooth muscle integrity as well as hyperplasia and hypertrophy of endothelial cells after VEGF treatment. Conceivably increased cavernous neovascularity may lead to functional or structural changes in the nerves and smooth muscles. Alternatively the direct effect of VEGF on the nerves and smooth muscle may also have a role since VEGF has been reported to have a direct trophic effect on the penile smooth muscle cells and spinal neurons in culture.^{28, 36, 37}

This pilot study was designed to examine the effect of VEGF administered minutes after arterial ligation. Additional studies are currently under way to examine whether VEGF can also be effective when given weeks after arterial ligation. We plan also to examine the effect of VEGF in chronic erectile dysfunction animal models, such as diabetic and hypercholesterolemic rats. In addition, the optimal dose, dosing interval and side effects must be determined in future studies. Briefly, our data show that ligation of the internal iliac arteries produces a reliable animal model of traumatic arteriogenic erectile dysfunction. A bolus injection of 4 μg VEGF into the corpus cavernosum after ligation of the internal iliac arteries seems to protect the penile tissue from ischemic insults and promote the recovery of erectile function.

CONCLUSIONS

Vasculogenic erectile dysfunction is one of the most common types of erectile dysfunction in clinical practice. In a rat model of traumatic erectile dysfunction created by bilateral ligation of the internal iliac arteries administration of a bolus of VEGF by intracavernous injection restored erectile function in a dose dependent fashion. In ligation plus PBS treated rats alterations in the myelinated and nonmyelinated nerves as well as in smooth muscle cells was evident compared with sham operated rats. VEGF treatment produced hyperplasia and hypertrophy of the endothelial cells, and restored the integrity of the nerves and smooth muscles. Further studies of the mechanism of VEGF function, optimal dose and frequency of treatment are warranted.

Ivy Hsieh, Electron Microscopy Laboratory and Dr. Benedict Yen, Department of Pathology, Veterans Administration Hospital, San Francisco assisted with electron microscopy studies and interpretation.

REFERENCES

1. Goldstein, I., Feldman, M. I., Deckers, P. J. et al: Radiation-associated impotence. A clinical study of its mechanism. *JAMA*, 251: 903, 1984
2. Rosen, M. P., Greenfield, A. J., Walker, T. G. et al: Arteriogenic impotence: findings in 195 impotent men examined with selective internal pudendal angiography. *Radiology*, 174: 1043, 1990
3. Levine, F. J., Greenfield, A. J. and Goldstein, I.: Arteriographically determined occlusive disease within the hypogastric-cavernous bed in impotent patients following blunt perineal and pelvic trauma. *J Urol*, 144: 1147, 1990
4. Heaton, J. P., Evans, H., Adams, M. A. et al: Coronary artery bypass graft surgery and its impact on erectile function: a preliminary retrospective study. *Int J Impot Res*, 8: 35, 1996
5. D'Amore, P. A. and Thompson, R. W.: Mechanisms of angiogenesis. *Annu Rev Physiol*, 49: 453, 1987
6. Folkman, J., Haudenschild, C. C. and Zetter, B. R.: Long-term culture of capillary endothelial cells. *Proc Natl Acad Sci USA*, 76: 5217, 1979
7. Gross, J. L., Moscatelli, D. and Rifkin, D. B.: Increased capillary endothelial cell protease activity in response to angiogenic stimuli in vitro. *Proc Natl Acad Sci USA*, 80: 2623, 1983

8. Anthony, F. W., Wheeler, T., Elcock, C. L. et al: Short report: identification of a specific pattern of vascular endothelial growth factor mRNA expression in human placenta and cultured placental fibroblasts. *Placenta*, **15**: 557, 1994
9. Neufeld, G., Cohen, T., Gengrinovitch, S. et al: Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J*, **13**: 9, 1999
10. Lei, J., Jiang, A. and Pei, D.: Identification and characterization of a new splicing variant of vascular endothelial growth factor: VEGF183. *Biochim Biophys Acta*, **1443**: 400, 1998
11. Jingjing, L., Xue, Y., Agarwal, N. et al: Human Müller cells express VEGF183, a novel spliced variant of vascular endothelial growth factor. *Invest Ophthalmol Vis Sci*, **40**: 752, 1999
12. Cheung, C. Y., Singh, M., Ebaugh, M. J. et al: Vascular endothelial growth factor gene expression in ovine placenta and fetal membranes. *Am J Obstet Gynecol*, **173**: 753, 1995
13. Burchardt, M., Burchardt, T., Chen, M. W. et al: Expression of messenger ribonucleic acid splice variants for vascular endothelial growth factor in the penis of adult rats and humans. *Biol Reprod*, **60**: 398, 1999
14. Hopkins, S. P., Bulgrin, J. P., Sims, R. L. et al: Controlled delivery of vascular endothelial growth factor promotes neovascularization and maintains limb function in a rabbit model of ischemia. *J Vasc Surg*, **27**: 886, 1998
15. Asahara, T., Bauters, C., Pastore, C. et al: Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation*, **91**: 2793, 1995
16. Hariawala, M. D., Horowitz, J. R., Esakof, D. et al: VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. *J Surg Res*, **63**: 77, 1996
17. Bauters, C., Asahara, T., Zheng, L. P. et al: Recovery of disturbed endothelium-dependent flow in the collateral-perfused rabbit ischemic hindlimb after administration of vascular endothelial growth factor. *Circulation*, **91**: 2802, 1995
18. Takeshita, S., Zheng, L. P., Brogi, E. et al: Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest*, **93**: 662, 1994
19. Bauters, C., Asahara, T., Zheng, L. P. et al: Physiological assessment of augmented vascularity induced by VEGF in ischemic rabbit hindlimb. *Am J Physiol*, **267**: H1263, 1994
20. Takeshita, S., Pu, L. Q., Stein, L. A. et al: Intramuscular administration of vascular endothelial growth factor induces dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia. *Circulation*, **90**: II228, 1994
21. Takeshita, S., Rossow, S. T., Kearney, M. et al: Time course of increased cellular proliferation in collateral arteries after administration of vascular endothelial growth factor in a rabbit model of lower limb vascular insufficiency. *Am J Pathol*, **147**: 1649, 1995
22. Banai, S., Jaklitsch, M. T., Shou, M. et al: Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation*, **89**: 2183, 1994
23. Baumgartner, I., Pieczek, A., Manor, O. et al: Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation*, **97**: 1114, 1998
24. Losordo, D. W., Vale, P. R., Symes, J. F. et al: Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation*, **98**: 2800, 1998
25. El-Sakka, A., Yen, T. S. and Lue, T. F.: Traumatic erectile dysfunction: a rat model. *Int J Impotence Res*, **13**: 162, 2001
26. Azadzi, K. M. and Saenz de Tejada, I.: Hypercholesterolemia impairs endothelium-dependent relaxation of rabbit corpus cavernosum smooth muscle. *J Urol*, **146**: 238, 1991
27. Kim, J. H., Klyachkin, M. L., Svendsen, E. et al: Experimental hypercholesterolemia in rabbits induces cavernosal atherosclerosis with endothelial and smooth muscle cell dysfunction. *J Urol*, **151**: 198, 1994
28. Liu, X., Lin, C.-S., Graziottin, T. et al: Vascular endothelial growth factor promotes proliferation and migration of cavernous smooth muscle cells. *J Urol*, **166**: 354, 2001
29. Magovern, C. J., Mack, C. A., Zhang, J. et al: Regional angiogenesis induced in nonischemic tissue by an adenoviral vector expressing vascular endothelial growth factor. *Hum Gene Ther*, **8**: 215, 1997
30. Folkman, J. and Shing, Y.: Angiogenesis. *J Biol Chem*, **267**: 10931, 1992
31. Ferrara, N.: The role of vascular endothelial growth factor in pathological angiogenesis. *Breast Cancer Res Treat*, **36**: 127, 1995
32. Walder, C. E., Errett, C. J., Bunting, S. et al: Vascular endothelial growth factor augments muscle blood flow and function in a rabbit model of chronic hindlimb ischemia. *J Cardiovasc Pharmacol*, **27**: 91, 1996
33. Harada, K., Friedman, M., Lopez, J. J. et al: Vascular endothelial growth factor administration in chronic myocardial ischemia. *Am J Physiol*, **270**: H1791, 1996
34. Dvorak, H. F., Nagy, J. A., Feng, D. et al: Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. *Curr Top Microbiol Immunol*, **237**: 97, 1999
35. Houck, K. A., Leung, D. W., Rowland, A. M. et al: Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem*, **267**: 26031, 1992
36. Jin, K. L., Mao, X. O. and Greenberg, D. A.: Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. *Proc Natl Acad Sci USA*, **97**: 10242, 2000
37. Sondell, M., Sundler, F. and Kanje, M.: Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. *Eur J Neurosci*, **12**: 4243, 2000

EXHIBIT B

Intracavernosal vascular endothelial growth factor (VEGF) injection and adeno-associated virus-mediated VEGF gene therapy prevent and reverse venogenic erectile dysfunction in rats

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Penile veno-occlusive dysfunction (venogenic erectile dysfunction) is a common cause of erectile dysfunction (ED). We investigated whether vascular endothelial growth factor (VEGF) can be used to prevent and reverse venogenic ED in a rat model. Pharmacological cavernosometry was developed and validated using adult male rats with either arteriogenic or venogenic ED. Castrated animals were treated with intracavernous VEGF as either a recombinant protein (C+VEGF) or adeno-associated virus (AAV)-mediated VEGF gene therapy (C+VEGF gene) in an attempt to prevent the development of venogenic ED. Other animal groups received testosterone replacement (C+testosterone) or intracavernous AAV-LacZ gene (C+LacZ). Animals with documented venogenic ED were treated with intracavernous VEGF in an attempt to reverse their ED. Functional analysis (pharmacological infusion cavernosometry) was performed following treatment. Penile specimens were harvested for immunohistochemistry and electron microscopic evaluation. Castrated rats showed a decrease in papaverine-induced intracavernous pressure and an increase in maintenance and drop rates during pharmacological cavernosometry. These changes were prevented by systemic testosterone and intracavernous VEGF or AAV-VEGF therapy. Moreover, intracavernous VEGF was able to reverse the venogenic ED produced by castration. The quantity of penile smooth muscle detected by alpha actin staining decreased after castration but not in the C+T, C+VEGF, or C+VEGF gene groups. Transmission electron microscopy revealed atrophy of penile smooth muscle cells and nerves in the castrated rats. In VEGF-treated rats, regeneration of smooth muscle and nerves as well as endothelial cell hypertrophy and hyperplasia were the prominent features. In our animal model, systemic testosterone replacement or intracavernous VEGF (protein and VEGF gene) prevented the veno-occlusive dysfunction in castrated animals. In rats with established venous leakage, VEGF treatment reversed the cavernosometric findings of leakage. Intracavernous injection of either VEGF protein or VEGF gene may be a preferred therapy to preserve erectile function in patients in whom testosterone therapy is contraindicated.

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Introduction

Following sexual arousal and release of nitric oxide to the erectile tissue, three events must take place to ensure an erection: trabecular smooth muscle re-

laxation, arterial dilation and venous compression.¹ During erection, blood filling of sinusoidal spaces compresses subtunical venules, thereby reducing venous outflow. With normal erectile function, a high intracavernous pressure (ICP) is maintained with a low inflow rate.² Patients with veno-occlusive dysfunction (venous leakage) exhibit a poor response to intracavernous injection with vasoactive agents (papaverine, prostaglandin E1, phentolamine, or combinations, for example), despite good arterial flow demonstrated by duplex ultrasound.

Following radical prostatectomy, approximately 30% of patients have vasculogenic erectile dysfunction.

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tion (ED) in addition to neurogenic ED and at least half of these men have venous leakage.³ Regardless of the etiology of organic ED (neurogenic, traumatic, hormonal, and vascular, etc), venous leakage is a common final condition resulting from smooth muscle atrophy.⁴ Furthermore, veno-occlusive dysfunction is the most common etiology of ED among nonresponders to medical management of ED.⁵

We propose that vascular endothelial growth factor (VEGF) may hold promise as a therapeutic agent for veno-occlusive dysfunction because of its known angiogenic effects and possible role as an androgen mediator. Following castration, rats do not display copulatory behavior or erectile function.⁶ They have depressed expression of nitric oxide synthase in the penis,⁷ undergo vascular involution in the ventral prostate⁸ and demonstrate decreased expression of VEGF in the prostate.⁹ Additionally, these animals display venogenic ED as assessed by ICP monitoring and blood flow studies.^{10,11} With testosterone repletion, VEGF synthesis is induced and followed by vascular regrowth in the ventral prostate of castrated rats,^{12,13} suggesting that VEGF may be the mediator of androgenic effects in the prostate. For this reason, we hypothesized that VEGF may also mediate androgenic effects in the penis and that VEGF administration may prevent ED after castration in rats.

The objectives of this study were (1) to establish normal values for pharmacologic cavernosometry in the rat and validate a rat model of venogenic ED (Experiment 1: Model validation), (2) to prevent venogenic ED (Experiment 2: Prevention trial), and (3) to reverse venogenic ED (Experiment 3: Treatment trial). Since the cavernosal sinusoids are lined with numerous endothelial cells, the target cell type of VEGF, the penis is an ideal organ for VEGF therapy. Our previous study also revealed that penile smooth muscle cells possessed VEGF receptors and VEGF treatment enhanced penile smooth cell proliferation and migration in culture.¹⁴ Additionally, VEGF has also been reported to be neuroprotective as well as neurotrophic.^{15,16} Since impairment of erectile nerves, endothelial cells, and the cavernous smooth musculature is the final common pathway of various types of organic ED, we conducted this study to examine whether VEGF can restore the integrity of the above-mentioned tissues and thus prevent or cure ED.

Experimental animals and methods

Animal groups

Male Sprague-Dawley rats aged 3–6 months (wt 350–450 g) were used in this study. They were housed in our animal care facility with rat chow

and water available *ad libitum* on a 12 h light/dark cycle. All animal care, treatments, and procedures were approved by the Committee on Animal Research at our institution. Rats were randomly divided for the animal model of vasculogenic ED (Experiment 1) and the VEGF prevention trial (Experiment 2). For the VEGF treatment trial (Experiment 3), the animals underwent castration and then were treated with VEGF after venous leak was demonstrated, to evaluate the efficacy of VEGF treatment in reversing established venogenic ED.

Experiment 1: To determine normal values for rodent pharmacologic cavernosometry and validate the model of venogenic ED in the rat, vasculogenic ED was induced. Arterial insufficiency was produced after performing a bilateral ligation of the internal iliac arteries. The acute and chronic effects of arterial insufficiency were evaluated 7 days and 30 days after bilateral iliac artery ligation. Venogenic ED was induced by castration, and pharmacologic cavernosometry was performed 6 weeks after surgery. Control animals underwent a sham laparotomy and studied 6 weeks later.

Experiment 2: For the prevention trial of VEGF in rats with venogenic erectile ED, adult males were castrated and immediately treated with hormone, intracavernosal VEGF. Hormone replacement was accomplished using a subcutaneously placed testosterone-filled silastic implant, as previously described.¹⁷ A therapeutic testosterone serum titer was confirmed in this animal group by testosterone radioimmunoassay¹⁸ performed by the biomedical core lab at our institution. Intracavernous treatment with VEGF was administered using either recombinant VEGF protein or an adeno-associated virus vector expressing the VEGF gene (AAV-VEGF). Control animals received a silastic implant containing saline, an intracavernous injection of normal saline, or an adenovirus transfection vector expressing lacZ reporter gene without the VEGF gene (AAV-LacZ).

Experiment 3: The trial of VEGF treatment was performed in castrated animals that were shown, 6–8 weeks following castration, to develop venogenic erectile ED by pharmacologic cavernosometry. These animals were treated with intracavernous VEGF protein and then after 1 month cavernosometry repeated to measure the effect of VEGF treatment.

Animal treatments

Surgical preparation: Prior to all surgical procedures, animals received anesthesia consisting of isoflurane inhalation as preanesthetic followed by an intraperitoneal injection of sodium pentobarbital (40 mg/kg). After the animal was asleep, electric clippers were used to trim the ventral abdominal

hair and the skin was prepped with chlorhexidine scrub. Antiseptic technique was maintained for all procedures. Following surgery, the anterior abdominal fascia and skin were approximated with 4-0 silk suture, analgesic buprenorphine (0.5 mg/kg s.c.) was administered and the animal allowed to awaken covered with a heating pad. Euthanasia was accomplished by an intraperitoneal injection of sodium pentobarbital (200 mg/kg), followed by bilateral thoracotomy when the animal was fully asleep.

Arterial ligation surgery: A 2 cm midline longitudinal low abdominal incision was made and a wheatlander retractor placed so that the plane between the prostate and sigmoid colon could be bluntly opened. Under a dissecting microscope with $\times 2.5$ –10 objectives, using sterile cotton-tipped swabs, the iliac artery bifurcation was identified and the common iliac exposed to the external iliac take-off. The internal iliac arteries were identified as those medial branches off the common iliac between the iliac bifurcation and the take-off of the external iliac. These were doubly ligated using 7-0 nylon sutures. After this was performed on both the right and left side, the incision was closed and the animal recovered as noted above.

Castration: A 2 cm midline longitudinal low abdominal incision was made and each testicle was grasped using forceps and brought into the incision. Each gubernaculum was divided using electrocautery and then the spermatic cords ligated with 4-0 silk suture and divided. After confirming hemostasis, the abdomen was closed and the rat recovered as above.

Intracorporal injections: A 1.5 cm oblique incision was made in the lower abdominal skin extending from the midline just above the penile hilum to below the level of the glans about 1 cm lateral to the midline. The skin was sharply dissected from the anterior surface of the penis and then the penis was retracted anteriorly using a towel clamp placed around it atraumatically with the foreskin left intact. Using blunt dissection the penile base and crura were exposed. The ischiocavernosus muscles were sharply dissected off the anterior surface of the crus until the white of the tunica albuginea of the corpora cavernosa was identified. The crus was then gently cannulated using a 23 gauge butterfly needle, and saline flush with a visual erectile response was used to confirm that the needle tip was truly intracavernosal. The intracavernosal injections were then administered with either VEGF protein (Calbiochem, Inc. La Jolla, CA, USA), AAV-VEGF, or AAV-LacZ. VEGF protein was administered at a dosage of 4 μ g/injection in 0.1 ml phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA). AAV-VEGF and AAV-LacZ constructs have been described previously¹⁹ and were administered at 10^{10} viral particles/injection in 0.1 ml PBS. Following injection, the needle was left in place for 5 min and then removed to allow the medication to diffuse

throughout the cavernosal space. Immediately thereafter, pinpoint electrocautery was applied to the needle hole for hemostasis and then the wound was closed and the animal recovered as above.

Testosterone replacement: Following castration, testosterone- or saline-filled silastic implants were placed in the subcutaneous tissue of the anterior abdominal wall, as previously described.¹⁷ Implants were prepared using sterile silastic tubing (Dow Corning, Midland, MI #602-265, inner diameter 0.062") that was filled with testosterone propionate powder (Sigma Chemical, St Louis, MO, USA) with the aid of wall suction. By radioimmunoassay,¹⁸ serum testosterone titer was found to be undetectable in the castrated animals and in the normal range for animals given testosterone implants.

Pharmacologic cavernosometry in the rat: To perform pharmacologic cavernosometry, both the right and left crura were separately cannulated using 23 gauge butterfly needles as described above. One cannula was flushed with sterile heparinized saline (100 U heparin/ml) and attached to a pressure detector for continuous intracorporal pressure (ICP) monitoring as previously described.¹¹ The contralateral cannula was attached to an infusion pump (Harvard Pump, Southwick, MA #55-2222), filled with sterile dilute heparinized saline (20 U heparin/ml). The baseline ICP was recorded (flaccid ICP) and then a dose of papavarine (1 mg in 0.1 ml) was administered through the infusion cannula. Overall 5 min was allowed for the papavarine to diffuse throughout the corpora and then the infusion cannula was flushed with heparinized saline and the pressure monitor cannula vented to normalize ICP after flushing. After another 5 min, the ICP was again recorded (ICP after papavarine) and the infusion started. An infusion rate of 0.05 ml/min was started and increased (by 0.05 ml/min every 10 s) until the ICP started to rise. Subsequent increases in inflow rate were made only after the ICP reached a plateau pressure. By slowly adjusting the inflow rate, an ICP of 100 cm H₂O (erectile pressure) was reached and the infusion rate required to maintain this pressure recorded (the maintenance rate). After this pressure was steady for 20 s, the infusion was terminated and the change in ICP over the subsequent 60 s was recorded (the drop rate).

Tissue preparation: After pharmacologic cavernosometry was performed, the penis was amputated at the crural bony attachments and immediately placed in ice-cold saline. The Y-shaped crura was sharply cut from the penile base and then a 1 mm thick slice cut for electron microscopy and placed in Karnofsky's solution (3% glutaraldehyde, 1% paraformaldehyde, 0.1 M sodium cacodylate buffer, pH 7.4). A 3 mm thick section of the distal penile shaft was then cut and placed in 10% normal buffered formalin for paraffin sections, and the balance of the penile shaft was flash frozen using dry ice in OCT

compound (Sakura Finetek USA, Torrance, CA, 3A) for frozen sectioning and immunohistochemistry.

Immunohistochemistry: Frozen sections were cut at 10 μ m, adhered to charged slides, air-dried for 15 min, and then rehydrated with 0.05 M PBS for 5 min. Sections were treated with hydrogen peroxide/methanol to quench endogenous peroxidase activity. After rinsing, sections were washed twice in PBS for 5 min, then incubated with 3% horse serum and 0.3% Triton X-100 at room temperature for 30 min. The serum solution was drained and then sections were incubated for 60 min with mouse monoclonal anti-alpha-smooth muscle actin (Sigma, St Louis, MO, USA) at a dilution of 1:4000 in PBS. After washing, sections were immunostained using the avidin-biotin-peroxidase method (Elite ABC, Vector Labs, Burlingame, CA, USA), with diaminobenzidine as the chromogen, followed by counterstaining with hematoxylin. Immunohistochemistry was performed in penile tissues from four rats randomly chosen from each subgroup.

Enzyme-linked immunosorbent assay: Serum samples from both systemic and penile blood were collected after whole-blood centrifugation. Solid-phase enzyme-linked immunosorbent assay for VEGF was performed using the Quantikine M mouse VEGF Immunoassay Kit (R&D Systems, Minneapolis, MN, USA) as previously described.¹⁴ Briefly, samples were diluted and added to microplate strip wells that were then treated with the enzyme-labeled immunoreactant VEGF conjugate. After incubation for 2 h and washing, the substrate solution was added and incubated for 30 min. The stop solution was added and then the optical density in each well determined using a microplate reader set to 450 nm. The results were plotted on a curve generated by the optical density of VEGF standards ranging from 0 to 500 pg/ml.

Transmission electron microscopy: The penis was dissected, thinly sliced (~1 mm thick), placed in Karnovsky's fixative (1% para-formaldehyde/3% glutaraldehyde/0.1 M sodium cacodylate buffer, pH 7.4) at room temperature for 30 min, and then stored at 4°C. The fixed tissue was then rinsed in buffer, postfixed in 2% aqueous OsO₄, and stained en bloc with uranyl acetate before being dehydrated in

ethanol, cleared in propylene oxide, and embedded in eponate12 (Ted Pella Co., Redding, CA, USA). Thick sections were cut and stained with toluidine blue, examined under light microscope to select the area to be thin sectioned. Thin sections were cut by Leica ultracut E microtome (Bannockburn, IL, USA), stained with uranyl acetate and Reynold's lead to enhance contrast and examined under Philips Tecnai 10 electron microscope (Eindhoven, the Netherlands).

Statistical analysis: We used computer software from Primer of Biostatistics, 3rd edn (Glantz SA, McGraw-Hill, Inc., New York, 1992) for statistical analysis. The data were first analyzed by one-way analysis of variance. If the difference was significant, Student-Neuman-Keuls test was used to perform the pairwise comparisons. A paired t-test was used where values represent findings before and after treatment in the same animals (Experiment 3, Table 3).

Results

Experiment 1: Model validation. The first goal of this study was to obtain normal values for pharmacologic cavernosometry in a rat model of vasculogenic ED. As shown in Table 1, flaccid ICP were comparable, in the range of 30 cm H₂O, among different groups of rats. However, after papavarine injection, control animals had a steep rise in ICP to >100 cm H₂O, while the castrated and ligated animals had lower responses. Only a minimal increase in ICP was noted in the animals following either castration or chronic internal iliac ligation, characteristic of vasculogenic ED. Acute ligation animals had a better response to papavarine than the chronic ligation group. After the infusion was started, both the control and acute ligation group promptly achieved erectile pressure with minimal inflow required. When the infusion was stopped, these animals had a minimal pressure drop, evidencing their intact veno-occlusive mechanism. The castration and chronic ligation groups, on the other hand, required a significantly higher infusion rate to maintain erectile pressure and experienced a steep pressure

Table 1 (Experiment 1) Cavernosometric findings in rat model of vasculogenic ED

	Flaccid ICP (cm H ₂ O)	ICP after papavarine (cm H ₂ O)	Maintenance rate (ml/min)**	Drop rate in 1 min (cm H ₂ O)**
Control (n=5)	35.4(±9.3)	104(±59)	0.024(±0.3)	9(±13)
Castration (n=7)	22.0(±5.2)	35.0(±5.0)	1.14(±0.5)	75(±5.4)
Acute ligation (n=6)	28.7(±7.6)	73.3(±10)	0.06(±0.12)	13.2(±13.8)
Chronic ligation (n=4)	29.3(±7.6)	38.3(±19)	1.9(±1.8)	45.0(±19)

* $P \leq 0.05$, control vs castration; control vs chronic ligation.

** $P \leq 0.05$, control vs chronic ligation; chronic ligation vs acute ligation.

*** $P \leq 0.05$, between all pair-wise comparisons except control vs acute ligation.

Table 2 (Experiment 2) Cavernosometric findings in castrated animals after prevention trial of testosterone replacement (C+testosterone), VEGF protein treatment (C+VEGF), AAV-VEGF gene therapy (C+VEGF gene), or LacZ control (C+LacZ control)

	Flaccid ICP (cm H ₂ O)	ICP after papavarine (cm H ₂ O) *	Maintenance rate (ml/min) **	Drop rate in 1 min (cm H ₂ O) ***
C+saline (n=5)	23.4(±5.3)	29.4(±15)	0.51(±0.26)	56.1(±15)
C+testosterone (n=7)	28.9(±7.5)	87.7(±26)	0.09(±0.1)	11.7(±16)
C+VEGF (n=5)	27.8(±5.2)	85.0(±28)	0.04(±0.09)	9.0(±20)
C+VEGF gene (n=11)	23.4(±7.1)	61.4(±36)	0.04(±0.03)	27.8(±18)
C+LacZ (n=5)	27.0(±8.7)	36.0(±12.8)	0.25(±0.31)	58.6(±8.1)

* $P \leq 0.05$, C+saline vs C+testosterone, C+VEGF; C+testosterone vs C+LacZ; C+VEGF vs C+LacZ.

** $P \leq 0.05$, C+saline vs C+testosterone, C+VEGF, and C+VEGF gene.

*** $P \leq 0.05$, C+saline vs C+testosterone, C+VEGF vs C+VEGF gene; C+LacZ vs C+VEGF, C+testosterone, and C+VEGF gene.

Table 3 (Experiment 3) Cavernosometric findings in VEGF treatment trial

	Flaccid ICP (cm H ₂ O)	ICP after Papavarine (cm H ₂ O)	Maintenance rate (ml/min)	Drop rate in 1 min (cm H ₂ O)
Before VEGF treatment (6 weeks after castration)	22.4(±6.9)	33.0(±12.3)	0.19(±0.18)	45.1(±18)
1 month following VEGF treatment	25.3(±8.5)	83.9(±31)	0.08(±0.15)*	17.4(±24)*

Animals (n=8) were castrated and then shown to have venous leak (after approximately 6 weeks) by pharmacologic cavernosometry. They were then treated with intracavernosal VEGF and 1 month later underwent repeat cavernosometry (* $P \leq 0.05$ for values before vs after VEGF treatment).

drop when the infusion was terminated. These findings are characteristic of venous leakage in the chronic ligation and castration groups.

Experiment 2: Prevention trial. Our second goal was to perform a prevention trial using intracavernosal VEGF either in the form of recombinant protein or AAV-mediated gene expression. As shown in Table 2, flaccid ICP was again in the range of 30 cm H₂O in each of the animal groups. After papavarine administration, however, both control groups (castration only and castration with LacZ injection) exhibited only weak rises in ICP, required a significant infusion rate to sustain an erectile ICP of 100 cm H₂O, and had a steep pressure drop after the inflow was terminated. In contrast, the three treatment groups exhibited nearly normal erectile function with high ICP in response to papavarine, a very low maintenance rate to sustain erectile ICP, and minimal pressure drop when the infusion was stopped. Of note, the VEGF gene-treated animals showed a lesser response to papavarine and a higher drop rate than the animals treated with either testosterone replacement or intracavernosal VEGF protein.

Experiment 3: Treatment trial. Our third goal was to perform a treatment trial using intracavernosal VEGF in animals with venous leak. Animals were castrated and, 4–6 weeks later, underwent cavernosometry. As shown in Table 3, before VEGF treatment, this animal group displayed a weak response to papavarine with ICP reaching 33 cm H₂O com-

pared to normal animals that attained nearly 100 cm H₂O (see Table 1). Also, these castrates required a relatively high maintenance rate (0.19 ml/min) to achieve erectile pressure and a steep drop rate when the infusion was terminated (45.1 cm H₂O in 60 s), evidencing venous leak. After these animals received intracorporal VEGF treatment, however, nearly normal erectile function returned with a prompt rise in intracorporal pressure after papavarine (to 84 cm H₂O), a low maintenance rate (0.08 ml/min) to achieve erectile pressure, and a minimal drop in ICP (17.4 cm H₂O in 60 s) after the infusion was terminated.

Immunohistochemistry: Quantitative analysis of the smooth muscle content (by alpha actin staining), as measured by computerized image analysis, produced the following pixel numbers: sham (43 518 ± 21 677), castrated (37 214 ± 18 814), castrated + testosterone treated (60 518 ± 18 733), castration + AAV-VEGF treated (51 690 ± 7 109), castrated + VEGF protein treated (52 990 ± 9 512). No significance between the groups was noted by one-way analysis of variance.

Transmission electron microscopy

Dorsal nerve. In sham-operated rats (Figure 1a), the dorsal nerve was filled with both myelinated and nonmyelinated nerve bundles. The mean diameter

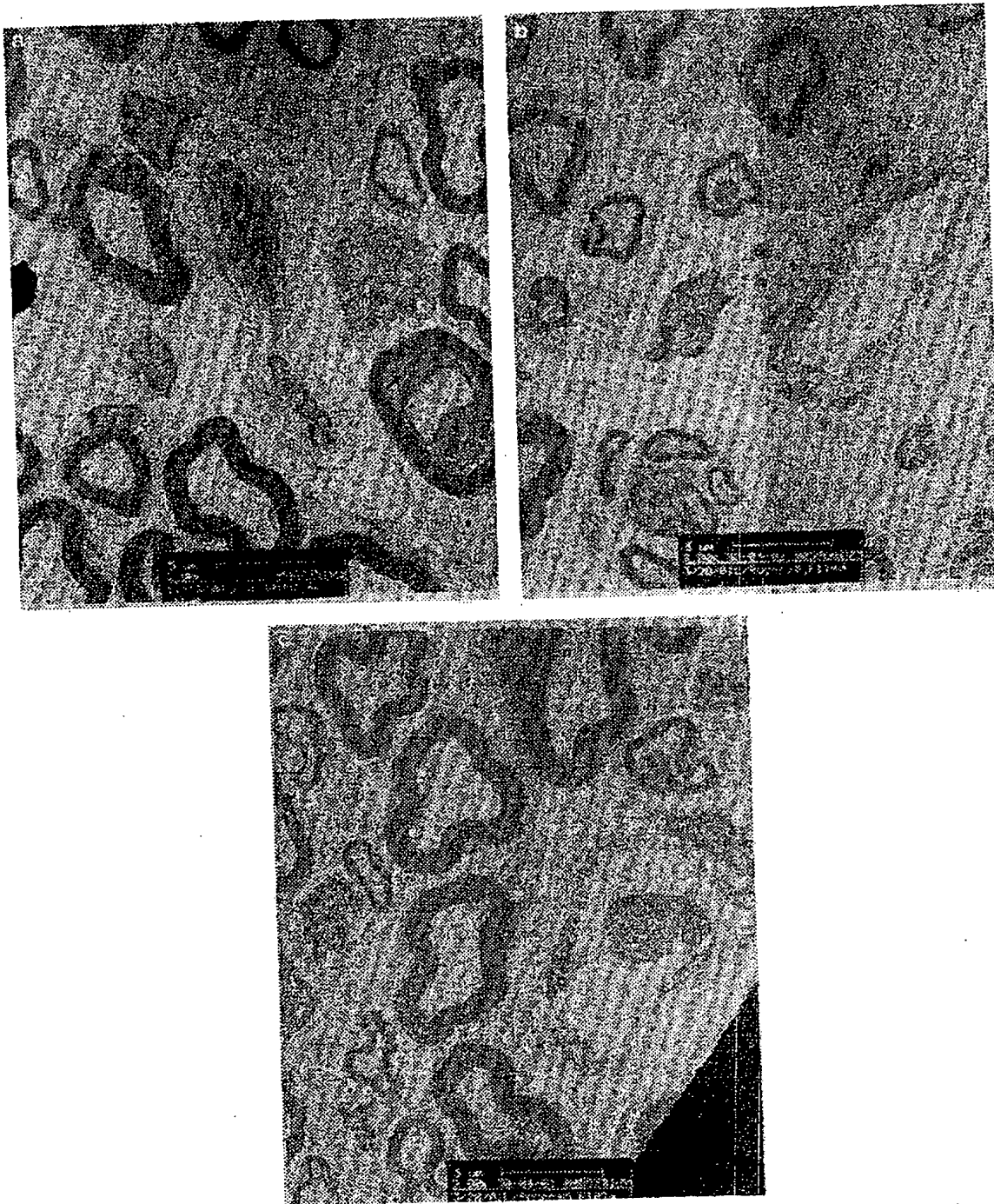


Figure 1 Representative electron micrographs of cross-sections of penile dorsal nerves ($\times 6500$). (a) Sham-operated rat. The dorsal nerve is filled with both myelinated (arrows) and nonmyelinated nerve fibers (arrow heads). The nuclei of Schwann cells are seen occasionally near the nerve fibers (curved arrow). (b) Castrated rat treated with LacZ. The diameter of the myelinated and nonmyelinated nerve fibers appear smaller than those of the sham-operated rats. Many nonmyelinated nerve fibers become indistinct and smaller. (c) Castrated rat treated with VEGF protein. Although many small myelinated nerve fibers are still present, larger fibers with thick myeline sheaths are also noted. The size of both myelinated and nonmyelinated nerve fibers appears larger than that of the castrated + Lac Z group.

of the individual myelinated axon (excluding myelin sheath) was $2.54 \pm 1.04 \mu\text{m}$. The mean thickness of the myelin sheath was $0.74 \pm 0.21 \mu\text{m}$. The mean diameter of the nonmyelinated axon was $0.97 \pm 0.35 \mu\text{m}$. The nuclei of Schwann cells were seen occasionally.

In castrated rats, with or without LacZ injection (Figure 1b), the diameter of both the myelinated and nonmyelinated axons appeared smaller than those of the sham-operated rats. Mean diameters were the following: myelinated axon $1.64 \pm 1.0 \mu\text{m}$; myelin sheath $0.49 \pm 0.13 \mu\text{m}$; nonmyelinated axon $0.64 \pm 0.32 \mu\text{m}$. Comparing the castrated rats to the sham-operated rats, the *P* values were 0.06, 0.004, and 0.001, respectively. Many nonmyelinated nerve fibers became indistinct and smaller. There was also an increase in the number of nucleated Schwann cells.

Although many small myelinated nerve fibers were still present in castrated rats treated with VEGF or AAV-VEGF (Figure 1c), larger fibers with thick myelin sheaths were also noted. The mean diameters of the myelinated nerve and myelin sheath were 2.36 ± 0.92 and $0.93 \pm 0.44 \mu\text{m}$, respectively. The nonmyelinated nerve fibers were more clearly defined but were not as abundant as the sham group. The mean diameter of nonmyelinated axons was $0.96 \pm 0.33 \mu\text{m}$. Comparing the VEGF-treated group to the castrated + Lac Z group, the *P* values of myelinated axon, myelin sheath, and nonmyelinated axon were 0.113, 0.05, and 0.000 respectively. The nerve fibers and myelin sheath in the testosterone replacement group appeared similar to the sham group.

Intracavernosal tissues

Intracavernous smooth muscle cells. In sham-operated rats, the smooth muscle cells (myocytes) were usually arranged in clusters and were separated by fine strands of fibroconnective tissue (Figure 2a). The cytoplasm of these myocytes contained abundant contractile myofilaments and dense bodies. Occasionally, small aggregates of organelles, including mitochondria, rough endoplasmic reticulum, and Golgi apparatus, were found adjacent to the nucleus. The cell membrane (sarcolemma) consisted

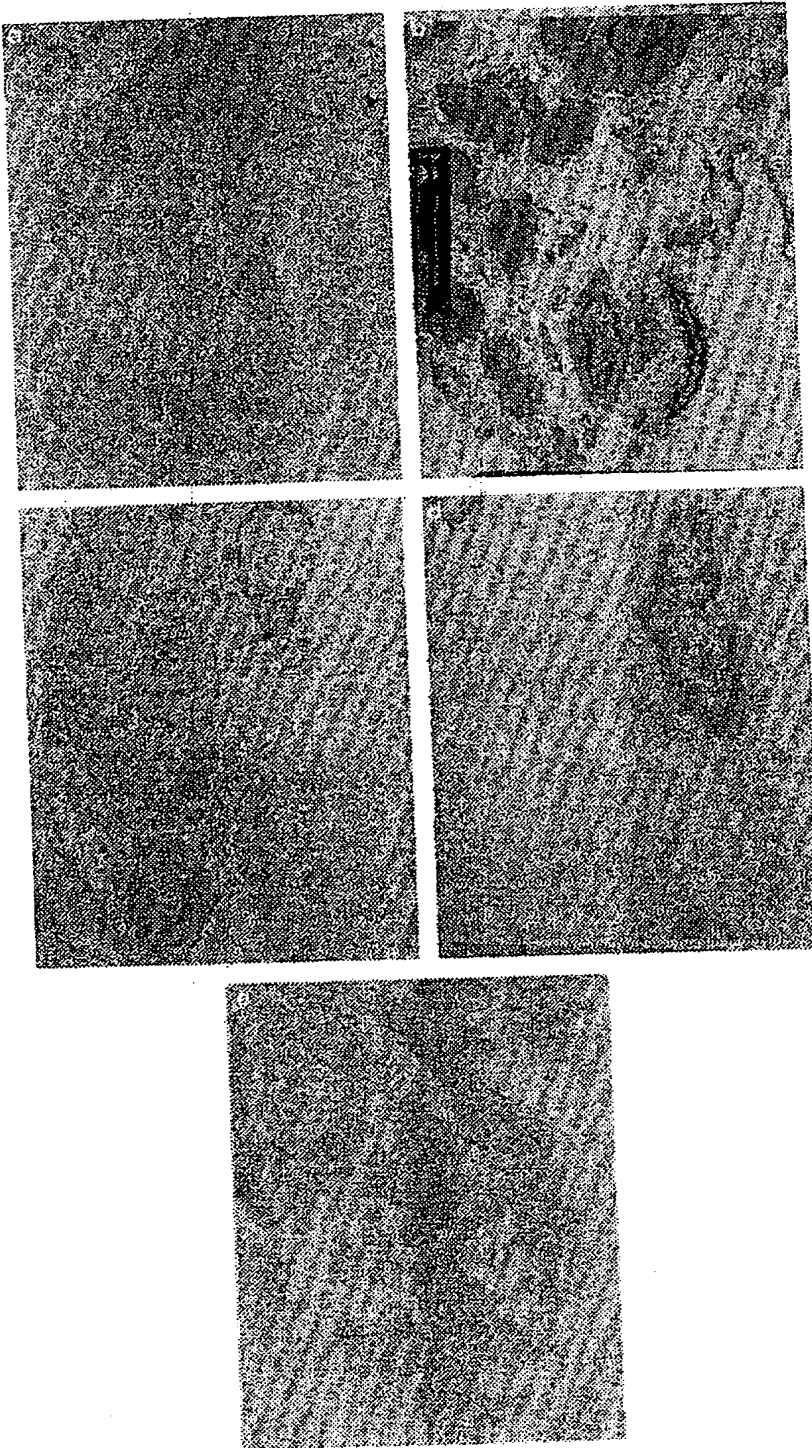
typically of alternating dense bands and light bands. The light bands contain numerous pinocytotic vesicles (caveolae). The intercellular spaces among myocytes were usually quite narrow with many gap junctions connecting individual cells. Nerve terminal varicosities were frequently seen located near clusters of smooth muscle cells. In low-power micrographs ($\times 6500$) of castrated rats with or without LacZ, the smooth muscle cells appeared scattered in a field of connective tissues (Figure 2b). The major differences between the castrated and castrated + testosterone rats were the increase in cytoplasmic myofilaments and the decrease in intercellular spaces in the latter group of rats. The myocytes in testosterone-treated rats appeared packed in clusters rather than scattered.

Striking differences were noted when comparing the AAV-VEGF and VEGF protein-treated rats to the castrated + Lac Z rats. The smooth muscles were arranged in clusters with minimal intercellular spaces (Figure 2c). Under high power ($\times 9400$), we noted the following: an increase in myofilaments and dense bodies, a decrease in dense bands, and an increase in the number of caveolae within the light bands of the sarcolemma (Figure 2d, e).

Endothelial cells. In sham-operated rats (Figure 3a), the cavernous sinusoids were lined by intact endothelium, the cytoplasm of which contained numerous pinocytotic vesicles (caveolae), mitochondria, rough endoplasmic reticulum, and Golgi apparatus. The nuclei of the endothelial cells were occasionally seen and appeared oval shaped or elongated. In castrated rats with or without LacZ, the appearance of the capillaries and cavernous sinusoidal endothelium was similar to the sham-operated group. In AAV-VEGF and VEGF-protein-treated rats, the nuclei of the endothelial cells lining most of the capillaries and sinusoids were plump and more numerous, indicative of endothelial hypertrophy and hyperplasia (Figure 3b, c).

Enzyme-linked immunosorbent assay: To examine whether the AAV-VEGF-treated animals had increased VEGF expression in the penis, blood samples were taken from the penis (penile blood following glans amputation) and from the abdominal aorta for animals treated with AAV-VEGF ($n = 7$) or AAV-LacZ ($n = 5$). While the mean VEGF titer in the systemic serum of animals that did not receive the VEGF gene (AAV-LacZ group) was $9.5 \pm 5.3 \text{ pg/}$

Figure 2 Representative electron micrographs of cross-sections of intracavernous erectile tissues. (a) Sham-operated rat ($\times 6500$). The smooth muscle cells (myocytes) are arranged in clusters and are separated by fine strands of fibroconnective tissue. The cytoplasm of these myocytes contained abundant contractile myofilaments. The cell membrane (sarcolemma) consists of alternating dense bands and light bands. The intercellular spaces among myocytes are narrow. (b) Castrated + AAV LacZ-treated rat ($\times 6500$). The smooth muscle cells (myocytes) appear scattered and separated by larger amounts of connective tissue. (c) Castrated + VEGF-protein-treated rat ($\times 6500$). The smooth muscles were arranged in clusters with minimal intercellular spaces. (d) Higher power microphotography of the intracavernous erectile tissue of a castrated + AAV LacZ-treated rat ($\times 21620$). The smooth muscle cells (myocytes) are separated by large amounts of connective tissue. The sarcolemma is lined mostly by dark bands. (e) Higher power microphotography of the intracavernous erectile tissue of a castrated + VEGF-protein-treated rat ($\times 21620$). The intercellular spaces are narrower than in (d). There is an increase in myofilaments and dense bodies in the cytoplasm. The alternating light and dark bands on the sarcolemma are more distinctive (arrow).



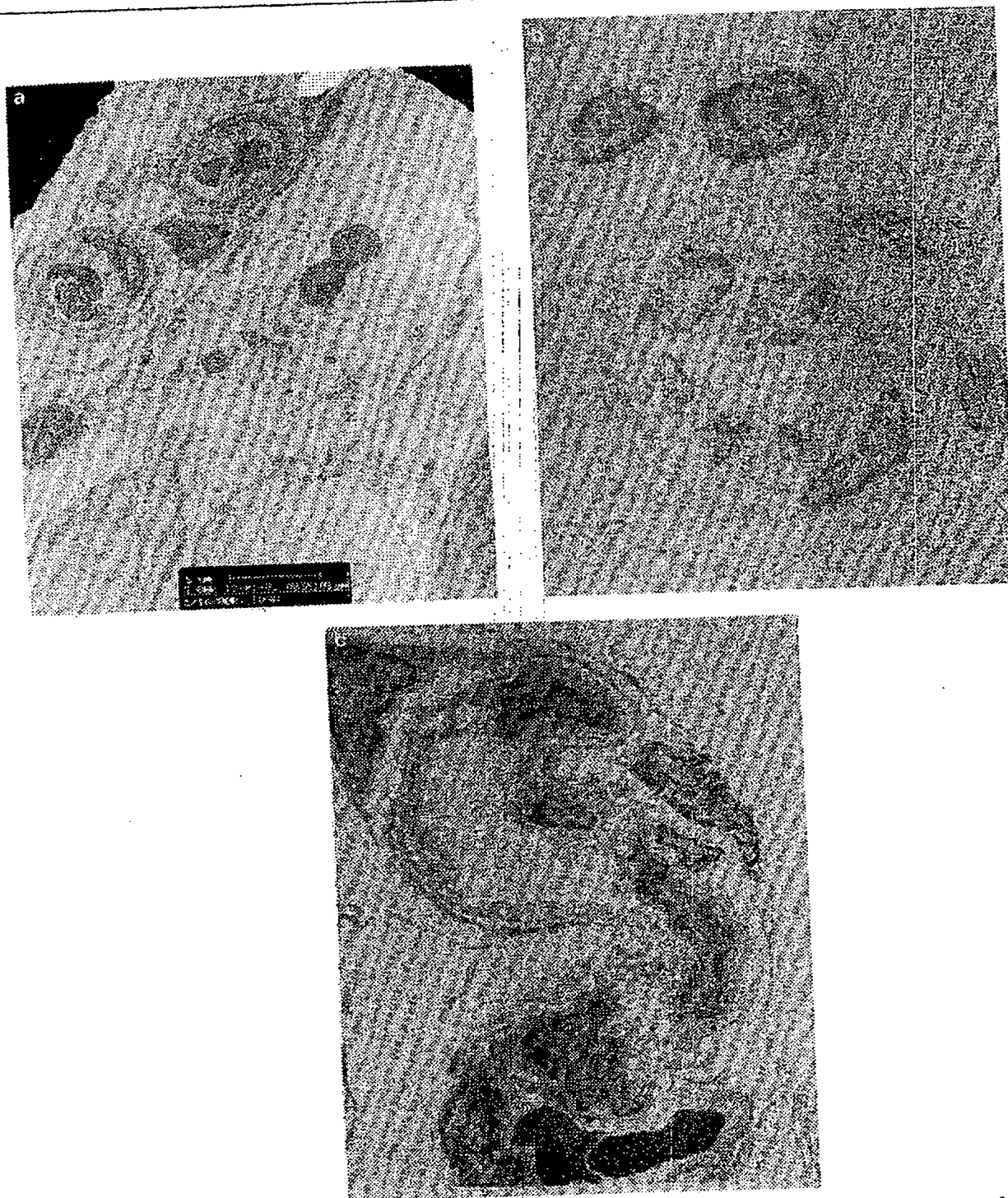


Figure 3 Representative electron micrographs of cross-sections of intracavernous penile tissues ($\times 6500$). (a) Castration + LacZ rats. Both the capillaries and cavernous sinusoids are lined by intact endothelium. The nuclei of the endothelial cells were occasionally seen (arrow) and appeared oval-shaped or elongated. In VEGF-protein-treated rats, the nuclei of the endothelial cells (arrows) lining many of the capillaries (b) and sinusoids (c) were large and more numerous, indicative of endothelial hypertrophy and hyperplasia.

ml, the AAV-VEGF-treated animals demonstrated a marked increase in VEGF titer at 23.3 ± 4.5 pg/ml ($P=0.04$). Similarly, serum from the penile blood in the AAV-LacZ group had a VEGF titer of 13.6 ± 4.7 pg/ml compared to a mean of 29.7 ± 6.8 pg/ml in the group receiving the intracavernosal VEGF gene ($P=0.039$). These differences are statistically significant, suggesting an increase of VEGF expression in the penile tissue after treatment with VEGF gene.

Discussion

Erectile function is a hemodynamic process of blood inflow and pressure maintenance in the cavernosal spaces.¹ As such, the penis is a predominantly vascular organ, and vascular insufficiency is the most common etiology of ED. Sinusoidal smooth muscle atrophy and collagen deposition are commonly found in men with long-standing ED of various etiologies, whether hormonal, neurological, or vascular.²⁰ Such degradation in smooth muscle quantity and quality leads to veno-occlusive dysfunction. This represents an end-stage muscular degeneration akin to myocardial changes with congestive heart failure or dilated cardiomyopathy for which no treatment currently exists.

Advancement in molecular biology has brought improved understanding of pathophysiology at the molecular level and offers treatment possibilities aimed at specific pathologic mechanisms. Treatment with VEGF in either protein or gene form has increased neovascularity in animal models of vasculopathies such as limb claudication²¹ and coronary artery disease.²² It has also been shown to improve symptomatic angina and wound healing in humans with inoperable heart disease and critical limb ischemia, respectively. The penis represents a convenient tissue target for gene therapy because of its external location, ubiquity of endothelial-lined spaces and low-level blood flow in the flaccid state. In addition, the penis is filled with billions of endothelial and smooth muscle cells, both of which are rich in VEGF receptors.¹⁴ In fact, three recent reports have shown beneficial effects of intracavernous VEGF therapy in animals with arteriogenic ED.²³⁻²⁵ The goal of the current study was to evaluate the efficacy of VEGF treatment in preventing and reversing the development of venogenic ED.

To this end, we first developed an animal model of venogenic ED by castration, as Mills et al^{10,11,26} have previously shown that castration leads to venogenic ED in rats within 6-8 days and testosterone repletion had a preventive effect. In these earlier studies ganglionic electrostimulation was used to generate an erection and the penile response was gauged with cavernosometric monitoring of ICP¹¹ or Doppler measurement of penile arterial inflow.¹⁰ Our goal was to devise a technique for evaluating

venous leak in animals similar to the technique used in humans. For this reason, erection was generated using pharmacologic agents (papavarine) instead of ganglionic electrostimulation. The physiologic parameters (maintenance inflow rate and ICP drop rate) used to diagnose venous leak in humans^{27,28} were reproduced in a rat model. Using this technique, pharmacocavernosometric findings were determined in normal animals and animals with venogenic and arteriogenic ED. This method was found to be a sensitive and reproducible technique to evaluate penile arterial insufficiency and venous leak in a rat model.

This model was then used to evaluate the efficacy of VEGF, administered intracorporally as recombinant protein or AAV gene vector, to prevent the development of venogenic erectile ED (Experiment 2: Prevention trial). It has been previously shown that castration induces an involution of the prostate gland and its vasculature.¹² Furthermore, after testosterone replacement, endothelial cell proliferation is stimulated and both blood flow and vascular volumes are normalized. After castration, prostatic VEGF synthesis is downregulated, as determined by RT-PCR, Western blot, and immunohistochemical analysis.¹³ Also, testosterone induces VEGF synthesis, suggesting that VEGF may be a tissue mediator of androgenic effects on the prostate. The goal of Experiment 2 was to determine if VEGF could prevent the development of venous leak in the rat model. Both testosterone replacement and VEGF treatment maintained erectile function when administered immediately after castration. Animal groups receiving no testosterone replacement or intracorporal AAV-LacZ showed persistent venogenic erectile ED after castration. Histological examination of smooth muscle content and morphology revealed deterioration in both the quality and quantity of penile smooth muscle after castration. Electron microscopic examination also revealed alteration of cell membrane and widening of intercellular spaces. Smooth muscle content as measured by alpha actin staining was normalized in animals receiving either testosterone or VEGF, evidence of preserved smooth muscle integrity with such preventative treatment.

The final phase was a treatment trial (Experiment 3) in which animals were first documented to have venous leak, 6 weeks after castration, and then treated with intracorporal VEGF protein. Cavernosometry was repeated and restoration of near normal erectile function was found 1 month after such treatment. We believe that this is the first experimental evidence of any medical therapy improving venogenic erectile ED. The exact mechanism by which VEGF improves erectile function is unknown. Nevertheless, we observed clear evidence of restoration of neural and smooth muscle integrity as well as hyperplasia and hypertrophy of endothelial cells after VEGF treatment. Conceivably, in-

creased cavernosal neovascularity may lead to functional or structural changes in the nerve and smooth muscles. Alternatively, the direct effect of VEGF on the nerve and smooth muscle may also play a role since VEGF has been reported to have a direct trophic effect on the penile smooth muscle cells¹⁴ and spinal neurons in culture.^{15,16} Further studies are under way to study the mechanism of VEGF action in the penis.

Conclusions

The technique presented here for pharmacologic cavernosometry is a simple and reproducible method to evaluate vasculogenic ED in a rat model. Normal erectile function and ED because of arterial insufficiency or venous leak may be diagnosed by characteristic cavernosometric findings. Using this technique, the presence of veno-occlusive disease may be diagnosed in animals 6 weeks after either castration or ligation of the internal iliac arteries.

Animals treated with testosterone replacement at the time of castration retain normal erectile function, while those without testosterone replacement develop venous leak. If these animals are treated with intracavernosal recombinant VEGF protein or AAV-VEGF at the time of castration, their erectile function is maintained and venous leak is prevented. The mechanism for this is not known at present, but we find a decrease in penile smooth muscle content in the castrated group compared with either the testosterone replacement group or the group receiving intracavernosal VEGF or AAV-VEGF. Penile smooth muscle morphology is uniformly degenerated after castration. Animals treated with the intracorporal AAV-VEGF transfection vector demonstrate significantly more VEGF protein in their penile serum compared to the systemic serum, and markedly more than control animals, indicating an increased expression of penile VEGF in these animals.

When rats with established venogenic ED are treated with one dose of intracavernosal recombinant VEGF protein, their erectile function returns to nearly normal, with reversal of the veno-occlusive defect. Electronmicroscopy revealed endothelial cell hyperplasia and hypertrophy as well as restoration of smooth muscle and neural integrity in the penile tissue after VEGF treatment. Since impairment of erectile nerve, endothelial cell, and the cavernous smooth musculature is the final common pathway of various types of organic ED, VEGF therapy may hold the key to prevention and cure of many forms of ED.

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References

- 1 Lue TF. *Contemporary Diagnosis and Management of Male Erectile Dysfunction*. Handbooks in Health Care Co.: Newtown, Penn., 1999.
- 2 Karadeniz T et al. Judgment of color Doppler ultrasound with respect to cavernous artery occlusion pressure in dynamic infusion cavernosometry when evaluating arteriogenic impotence. *Urol Int* 1996; 57: 85–88.
- 3 Mulhall JP, Graydon RJ. The hemodynamics of erectile dysfunction following nerve-sparing radical retropubic prostatectomy. *Int J Impot Res* 1996; 8: 91–94.
- 4 Mersdorf A et al. Ultrastructural changes in impotent penile tissue: a comparison of 65 patients. *J Urol* 1991; 145: 749–758.
- 5 Rajfer J, Rosciszewski A, Mebrieger M. Prevalence of corporeal venous leakage in impotent men. *J Urol* 1988; 140: 69–71.
- 6 Meisel RL, O'Hanlon JK, Sachs BD. Differential maintenance of penile responses and copulatory behavior by gonadal hormones in castrated male rats. *Horm Behav* 1994; 18: 56–64.
- 7 Zvara P et al. Nitric oxide mediated erectile activity is a testosterone dependent event: a rat erection model. *Int J Impot Res* 1995; 7: 209–219.
- 8 Lekäs E et al. Decrement of blood flow precedes the involution of the ventral prostate in the rat after castration. *Urol Res* 1997; 25: 309–314.
- 9 Häggström S, Wikström P, Bergh A, Damber JE. Expression of vascular endothelial growth factor and its receptors in the rat ventral prostate and Dunning R3327 PAP adenocarcinoma before and after castration. *Prostate* 1998; 36: 71–79.
- 10 Mills TM, Lewis RW, Stopper VS. Androgenic maintenance of inflow and veno-occlusion during erection in the rat. *Biol Reprod* 1998; 59: 1413–1418.
- 11 Mills TM, Stopper VS, Wiedmeier VT. Effects of castration and androgen replacement on the hemodynamics of penile erection in the rat. *Biol Reprod* 1994; 51: 234–238.
- 12 Franck-Lissbrant I, Häggström S, Damber JE, Bergh A. Testosterone stimulates angiogenesis and vascular regrowth in the ventral prostate in castrated adult rats [see comments]. *Endocrinology* 1998; 139: 451–456.
- 13 Häggström S, Lissbrant IF, Bergh A, Damber JE. Testosterone induces vascular endothelial growth factor synthesis in the ventral prostate in castrated rats. *J Urol* 1999; 161: 1620–1625.
- 14 Liu X et al. Vascular endothelial growth factor promotes proliferation and migration of cavernous smooth muscle cells. *J Urol* 2001; 166: 354–360.
- 15 Jin KL, Mao XO, Greenberg DA. Vascular endothelial growth factor: direct neuroprotective effect in *in vitro* ischemia. *Proc Natl Acad Sci USA* 2000; 97: 10242–10247.
- 16 Sondell M, Sundler F, Kanjo M. Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the Trk-1 receptor. *Eur J Neurosci* 2000; 12: 4243–4254.
- 17 Verdonck A et al. Effect of testosterone replacement after neonatal castration on craniofacial growth in rats. *Arch Child Biol* 1998; 43: 551–557.
- 18 Parker CR Jr, Ellegood JO, Mahesh VB. Methods for multiple steroid radioimmunoassay. *J Steroid Biochem* 1975; 6: 1–8.
- 19 Su H, Lu K, Kan YW. Adeno-associated viral vector-mediated vascular endothelial growth factor gene transfer induces neovascular formation in ischemic heart. *Proc Natl Acad Sci USA* 2000; 97: 13801–13806.
- 20 Karadeniz T et al. Correlation of ultrastructural alterations in cavernous tissue with the clinical diagnosis vasculogenic impotence. *Urol Int* 1996; 57: 58–61.
- 21 Baumgartner I et al. Constitutive expression of pVEGF105 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia [see comments]. *Circulation* 1998; 97: 1114–1123.

- 22 Symes JF *et al*. Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. *Ann Thorac Surg* 1999; **68**: 830-836; discussion 836-837.
- 23 Henry GD *et al*. Intracavernosal injections of vascular endothelial growth factor protects endothelial dependent corpora cavernosal smooth muscle relaxation in the hypercholesterolemic rabbit: a preliminary study. *Int J Impot Res* 2000; **12**: 334-339.
- 24 Byrne RR *et al*. Vascular endothelial growth factor restores corporeal smooth muscle function *in vitro*. *J Urol* 2001; **165**: 1310-1315.
- 25 Lee MC *et al*. The effect of vascular endothelial growth factor on a rat model of traumatic arteriogenic erectile dysfunction. *J Urol* 2002; **167**: 761-767.
- 26 Mills TM, Dai Y, Stopper VS, Lewis RW. Androgenic maintenance of the erectile response in the rat. *Steroids* 1999; **64**: 605-609.
- 27 Hatzichristou DG *et al*. *In vivo* Assessment of trabecular smooth muscle tone, its application in pharmacocavernosometry and analysis of intracavernous pressure determinants [see comments]. *J Urol* 1995; **153**: 1126-1135.
- 28 Karadeniz T *et al*. Value of color Doppler sonography in the diagnosis of venous impotence. *Urol Int* 1995; **55**: 143-146.